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13. ABSTRACT (Maximum 200 words) Understanding the interactions between neurons and their denervated targets is critical to developing techniques to improve reinnervation of the denervated following nerve injury, and for attempting to facilitate regeneration in the central nervous system (CNS) where it does not occur. Experiments were designed to determine whether target-derived factors direct the outgrowth or regenerating axons and their own reinnervation. We have shown that neurite outgrowth from both sensory and motor neurons is directed up concentration gradients of target-derived factors both in vitro and in vivo. Using these gradients and the cells that secrete the factors we have succeeded in promoting regeneration across peripheral nerve gaps of up to 2 cm. Experiment are underway to bridge gaps of up to 8 cm. The target-derived factors that promote and direct axon regeneration, as well as determine the morphology of the processes are being characterized (more than 6 have been demonstrated). The presence of a peripheral nervous system regeneration inhibiting factor has been demonstrated and methods developed to block its influence. Experiments are underway to determine				
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Title: Characterization of Neurotrophic and Neurotropic Interactions between Neurons and their Muscle and Nerve Targets

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Name of Institution: Institute of Neurobiology, Medical Sciences Campus, Univ. of Puerto Rico

Authors of Report: Damien Kuffler, Ph.D.

List of Manuscripts in Preparation

Kuffler, D.P. Inhibition of neurite outgrowth in the peripheral nervous system.

Betancourt, A., & D.P. Kuffler. Blocking peripheral nervous system neurite outgrowth inhibition with neurotrophic factors.

Betancourt, C. Sekirnjak, A. Rivalta, A., Q. Zhao, & D.P. Kuffler. Promoting axon regeneration across a 3 cm gap via seeded peripheral nerve cells in a silicon tube.

Hill, E., G. Latalladi, & D.P. Kuffler. Diffusible concentration gradients of target-derived factors direct motor axon regeneration in vitro.

Zheng, M. & D.P. Kuffler. Regenerating axons in vivo growth up concentration gradients of target-derived factors.

Scientific Personnel Supported:

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Report of Inventions by Title: None

Statement of Problems Studied:

1. Do concentration gradients of peripheral nerve-released factors direct the growth cone sensory and motor neuron growth cones in vitro?
2. Do concentration gradients of peripheral nerve-released factors direct the growth motor axons in vivo?

3. Can novel methods be developed to facilitate regeneration across long gaps in peripheral nerves?
4. What factors are required to protect adult human neurons from neurotoxicity following spinal trauma?
5. Characterization of peripheral nerve-released factors that promote neurite outgrowth and modify neurite morphology.
6. Are there regeneration inhibiting factors in the peripheral nervous system?
7. What role does innervation have on the maintenance of skeletal muscle fiber subsynaptic nuclei clustering?
8. What influences do skeletal muscle fiber-released factors exert on motoneuron process outgrowth?
9. What are the membrane properties of adult sensory and motor neurons?

Summary of Most Important Results

1. Do concentration gradients of peripheral nerve-released factors direct the growth cone sensory and motor neuron growth cones in vitro?

We have demonstrated diffusible that concentration gradients of peripheral nerve-derived factors reliably direct the outgrowth of sensory (28/28) and motor neuron (47/47) growth cones in vitro. This influence is exerted over gradients up to 200 μ m long.

2. Do concentration gradients of peripheral nerve-released factors direct the growth motor axons in vivo?

Concentration gradients of peripheral nerve-derived factors reliably direct the outgrowth of motor axons in vivo. This influence is exerted over gradients up to 6.5 mm.

3. Can novel methods be developed to facilitate regeneration across long gaps in peripheral nerves?

The central and distal nerve stumps of a peripheral nerve have been inserted into silicon tubes filled with bioresorbable matrices into which are injected dissociated cells of a peripheral nerve. Using these bridging tubes peripheral axons can now be made to successfully regenerate across gaps of more than 2 cm.

4. What factors are required to protect adult human neurons from neurotoxicity following spinal trauma?

Using methods developed in this laboratory, adult human sensory neurons have been isolated and maintained in culture for more than 8 weeks where they extend neurites and remain electrically excitable. The best survival of such isolated adult neurons by other laboratories is 24 hours. Normally such neurons die due to the neurotoxic environment created during the isolation procedures or spinal trauma. The methods used to isolate the neurons are being analyzed to determine which components of the protocols are responsible for protecting the neurons from neurotoxicity.

5. Characterization of peripheral nerve-released factors that promote neurite outgrowth and modify neurite morphology.

We have demonstrated that medium conditioned by a length of peripheral nerve contains a number of factors (a minimum of 6), with vastly differing molecular weights, that promote neurite outgrowth from adult neurons and modify the morphology of the neurites. Although the responsible

factors have not been isolated, the molecular weights of factors in some fractions indicate that some of these factors are not part of the known neurotrophin family. These results also demonstrate multiple factors must be present and act simultaneously on adult neurons to promote neurite outgrowth and extensive elongation of the neurites, as well as to induce neurites with a physiological appropriate morphology.

6. Are there regeneration inhibiting factors in the peripheral nervous system?

We have demonstrated that satellite cells associated with adult rat sensory neurons inhibit neurite outgrowth. However, this inhibitory influence can be reduced by specific neurotrophic factors and completely blocked by a combination of peripheral nerve-released factors. The influence of the neurite outgrowth inhibitory factor appears to be most effective when the neurons are in contact with the satellite cells, however, the factor also appears to act in a diffusible manner on neurons not in contact with satellite cells. Preliminary results indicate that the factor is a heparan sulfate proteoglycan.

7. What role does innervation have on the maintenance of skeletal muscle fiber subsynaptic nuclei clustering?

Subsynaptic nuclei clustering in adult skeletal muscle fibers appears to result from nerve released factors that become bound to the muscle fiber extracellular matrix and potentially interact with integrin receptors to induce and maintain the nuclei clustering.

8. What influences do skeletal muscle fiber-released factors exert on motoneuron process outgrowth?

Skeletal muscle fibers release diffusible factors that promote and direct neurite out from cultured adult motoneurons.

9. What are the membrane properties of adult sensory and motor neurons?

The biophysical properties of adult sensory and motor neurons have been characterized using electrophysiological approaches. It was shown that several types of sensory neurons can be identified by their morphology and that each population has different membrane properties. This technique should allow for the identification of different populations of neurons based on morphology alone.

**List of All Publications and Technical Reports Published
Papers**

Kuffler, D.P. (1994). Promoting and Directing Axon Outgrowth. *Molecular Biology. Molec. Neurobiol.* 9:233-243.

Kuffler, D.P. & O. Megwinoff, (1994). Neurotrophic influence of denervated sciatic nerve on adult dorsal root ganglion neurons. *J. Neurobiol.* 25:1267-1282.

Dobretsov, M., A. Dobretsov, & D.P. Kuffler (1994). Influence of factors released from sciatic nerve on adult dorsal root ganglion neurons. *J. Neurobiol.* 25:1249-1266.

Skatchkov, S., C. Broesamle, L. Vyklicky, D.P. Kuffler & R.K. Orkand (1994). NMDA receptors on adult frog spinal motoneurons in culture. *Neurosci. Lett.* 176:251-254.

Philippi, M., L. Vyklicky, D.P. Kuffler & R.K. Orkand (1995). Serotonin and proton induced and modified ionic currents in frog sensory neurons. *J. Neurosci. Res.* 40:387-395.

Kuffler, D.P. (1996). Chemoattraction of sensory neuron growth cones by diffusible concentration gradients of acetylcholine. *Molec. Chem. Neuropath.* 28:199-208.

Kuffler, D.P. (1996). Chemotropic factors direct regenerating axons. *News in Phys. Sci.* 11:219-222.

Broesamle, C. & D.P. Kuffler (1996). Rapid dispersal of clustered synaptic nuclei upon dissociation of *Rana pipiens* skeletal muscle fibers. *J. expl. Biol.* 199:2359-2367.

Pérez, N.L., M.S. Sosa, & D.P. Kuffler (1997). Growth cones turn up concentration gradients of diffusible peripheral target-derived factors. *Exp. Neurol.* 145:196-202.

Xie, F., G. Latalladi, & D.P. Kuffler (1997). Neurotrophic influence of sciatic nerve-released factors on adult motor neurons *in vitro*. *J. Periph. Nerv. Sys.* 3:1-10.

Sosa, I., O. Reyes, A., Inserni, & D.P. Kuffler (1998). Isolation and Long-term Survival of Adult Human Sensory Neurons *in vitro*. *Neurosurgery* 42:681-686.

Abstracts

Pérez, N.L. & D.P. Kuffler (1994). Directing the outgrowth of regenerating axons. *Puerto Rico Health Sci. J.*

Broesamle, C. & D.P. Kuffler (1994). Neurotrophic influence of isolated adult skeletal muscle fibers on cultured motoneurons. *Puerto Rico Health Sci. J.*

Kuffler, D.P. (1994). Diffusible factors provide long-distance guidance for neuronal processes. *ARO Biosci. Conf., Specific Interactions of Biological Molecules.*

Skatchkov, S., C. Broesamle, L. Vyklicky, D.P. Kuffler & R.K. Orkand (1994). NMDA receptors on adult frog spinal motoneurons in culture. *Sixth Puerto Rico EPSCoR Annual Conf.* 6:29.

Kuffler, D.P. & N. Vélez (1994). Directing axon regeneration. *Sixth Puerto Rico EPSCoR Annual Conf.* 6:35.

Broesamle, C. & D.P. Kuffler (1994). Acetylcholine receptors on isolated muscle fibers *in vitro*. *Sixth Puerto Rico EPSCoR Annual Conf.* 6:34.

Kuffler, D.P. (1994). Growth cones are directed up concentration gradients by factors released from

- denervated peripheral nerve tubes. Soc. Neurosci. Abstr. 284.2.
- Kuffler, D.P. (1994). Growth cones elongate up concentration gradients of neurotransmitters and neurotrophins. Third Puerto Rico Neuroscience Conference, 8.
- Zheng, M. & D.P. Kuffler (1994). Do axons find their targets via diffusible tropic factors? Third Puerto Rico Neuroscience Conference, 7.
- Xie, F. & D.P. Kuffler (1994). Co-culture of adult frog muscle fibers and neurons. Third Puerto Rico Neuroscience Conference, 9.
- Broesamle, C. & D.P. Kuffler (1994). Redistribution of subsynaptic nuclei in frog skeletal muscle fibers after enzymatic isolation. Third Puerto Rico Neuroscience Conference, 6.
- Kuffler, D.P. (1995). Neuronal targets direct their own reinnervation via concentration gradients of diffusible factors. 3rd Symp.: Neurodegenerative Disorders: Common Molecular Mechanisms.
- Zheng, M. & D.P. Kuffler (1995). Do axons find their targets via diffusible tropic factors? RCM, UPR Research Forum, March 1995.
- Kuffler, D.P. (1995). Growth cones elongate up concentration gradients of neurotransmitters and neurotrophins. RCM, UPR Research Forum, March 1995.
- Broesamle, C. & D.P. Kuffler (1995). Redistribution of subsynaptic nuclei in frog skeletal muscle after enzymatic isolation. RCM, UPR Research Forum, March 1995.
- Xie, F. & D.P. Kuffler (1995). Co-cultures of adult frog muscle fibers and neurons. RCM, UPR Research Forum, March 1995.
- Xie, F.K., M. Zheng, & D.P. Kuffler (1995). Neurotrophic influence of denervated sciatic nerve on adult motoneurons. Soc. Neurosci. Abstr.
- Xie, F.K., M. Zheng, & D.P. Kuffler (1995). Neurotrophic influence of denervated sciatic nerve on adult motoneurons. 4th P.R. Ann. Neurosci. Conf. Abstr.
- Zheng, M., F.K. Xie, & D.P. Kuffler (1995). Regenerating axons grow up concentration gradients of diffusible target-derived factors *in vivo*. 4th P.R. Ann. Neurosci. Conf. Abstr.
- Hill, E. & D.P. Kuffler (1996). Diffusible gradients of target-derived factors direct axon regeneration. ARO Symposium: Biomolecular Signal Generators and Transduction Processes: Implications for sensors and other Device Applications. Cashiers, NC.
- Latalladi, G., E.S. Hill, & D.P. Kuffler (1996) Growth cones of isolated adult motoneurons turn up

concentration gradients of peripheral nerve-derived factors. 5th PR Neurosci. Conf.

Sosa, I., O. Reyes, A. Betancourt, & D.P. Kuffler (1997). Isolation and Long-term Survival of Adult Human Sensory Neurons *In Vitro*. Neurodegenerative Disorders: Common Molecular Mechanisms, Jamaica, February.

Sosa, I., O. Reyes, J. Lastre, A. Betancourt, J. Inserni, & D.P. Kuffler (1997). Improvements in methods for dissociation and long-term survival of isolated adult human sensory neurons *in vitro*. Ann. Meeting, Am. Soc. of Neurosurg., San Juan, PR., February.

Latalladi, G., E.S. Hill, & D.P. Kuffler (1997). The importance of target-derived factors in directing the precision of motor axon regeneration. PR Heal. Sci. J. A-96.

Hill, E.S. & D.P. Kuffler (1997). Tropic influence of muscle-derived factors on adult motor neurons *in vitro*. PR Heal. Sci. J. A-97.

Sekirnjak, C., I. Bonilla, A. Morales & D.P. Kuffler (1997). Using λ -shaped silicone chambers to study axon guidance *in vivo*. PR Heal. Sci. J. A-98.

Sosa, I., O. Reyes, J. Lastre, A. Betancourt, J. Inserni, & D.P. Kuffler (1997). Improvements in methods for dissociation and long-term survival of isolated adult human sensory neurons *in vitro*. EPSCoR / AAAS Annual Meeting, Fajardo, PR, May.

Latalladi, G., E.S. Hill, & D.P. Kuffler (1997). The importance of target-derived factors in directing the precision of motor axon regeneration. EPSCoR / AAAS Annual Meeting, Fajardo, PR, May.

Hill, E.S. & D.P. Kuffler (1997). Tropic influence of muscle-derived factors on adult motor neurons *in vitro*. EPSCoR / AAAS Annual Meeting, Fajardo, PR, May.

Sekirnjak, C., I. Bonilla, A. Morales & D.P. Kuffler (1997). Using λ -shaped silicone chambers to study axon guidance *in vivo*. EPSCoR / AAAS Annual Meeting, Fajardo, PR, May.

Sosa, I., O. Reyes, J. Lastre, A. Betancourt, J. Inserni, & D.P. Kuffler (1997). Improvements in methods for dissociation and long-term survival of isolated adult human sensory neurons *in vitro*. Ist International Inter Latin American Spinal Cord Society Meeting, SIALC, Fajardo, June.

Morales, A., S. Garcia, & D.P. Kuffler (1997). Multiple synergistically acting peripheral nerve-released neurotrophic factors. 6th PR Neuroscience Conference. Dec. 6, 6:36.

Rivalta, A., C. Sekirnjak & D.P. Kuffler (1997). Improved techniques for promoting peripheral nerve regeneration across long gaps *in vivo*. 6th PR Neuroscience Conference. Dec. 6, 6:19

Sekirnjak, C., & D.P. Kuffler (1997). Novel techniques for promoting and directing axon outgrowth.

Sixth Ann. Meeting Israel Soc. Neurosci., Eilat, Neurosci. Lett. 48:S44.

Kuffler, P.D. (1998). A peripheral nervous system model for CNS neurite outgrowth inhibition. 5th Symposium: Neurodegenerative Disorders: Common Molecular Mechanisms, Ocho Rios, Jamaica, Feb. 22-28, 1998

Kuffler, D.P., A. Morales, & L. Bonilla (1998). Inhibition of peripheral nervous system regeneration. UPR Res. Forum, PR Health Sci. J.

Morales, A, S. Garcia-Castiñeiras, & D.P. Kuffler (1998). Biochemical analysis of peripheral nerve-released factors. PR Health Sci. J.

Rivalta, A. F. Quiles, & D.P. Kuffler (1998). Promoting regeneration of peripheral nerves across long gaps in vitro. UPR Res. Forum, PR Health Sci. J.

Morales, A., S. Garcia-Castiñeiras, & D.P. Kuffler (1998). Multiple peripheral nerve-released factors act simultaneously to promote process outgrowth with unique morphologies. Soc. for Neurosci. Abstr.

Reyes, O., I. Sosa, & D.P. Kuffler (1998). Optimizing conditions for the survival of adult human neurons following trauma. 7th Puerto Rico Neuroscience Conference, Bayamon. Dec. 5.

Betancourt, A. & D.P. Kuffler (1998). Repair of long distance peripheral nerve gaps. 7th Puerto Rico Neuroscience Conference, Bayamon. Dec. 5.

Quiles, F. & D.P. Kuffler (1998). Specificity of reinnervation of distal nerve stumps by regenerating motor axons. 7th Puerto Rico Neuroscience Conference, Bayamon. Dec. 5.

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